A COMPARISON OF SALT LOSS RATE IN THREE SPECIES OF BRACKISH–WATER NEREID POLYCHAETES

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Different species of nereid polychaetes are able to regulate to varying degrees the salt concentration and osmotic pressure of the coelomic fluid in waters of low salinity (Schlieper, 1929; Beadle, 1937; Jørgensen and Dales, 1957; Smith, 1955). Hyper-regulation of coelomic salt concentration represents a steady-state in which losses of ions to the medium via body wall, nephridia, and gut are balanced by mechanisms of ion intake by body wall and/or gut. The tendency to lose body salts might be regarded as of negative adaptive value to a polychaete hyper-regulating in a situation of unchanging low salinity, since a high loss rate would impose a high load upon uptake mechanisms. On the other hand, the ability to lose salts rapidly might not be a disadvantage, and might be considered a favorable adaptation, in a polychaete subjected to a sudden lowering of environmental salinity, since by lowering the osmotic gradient the animal would be faced with a less severe problem of volume control and of disposing of excess water taken in osmotically. Indeed, it is likely that the process of effective volume control by urine output might contribute markedly to the lowering of internal salt concentration and so to the reduction of water intake to a point where mechanisms of volume control could be effective. Thus, while it might seem reasonable to expect to find a low tendency to lose salts in an estuarine or fresh-water polychaete, there are also reasons why an effective salt-losing mechanism could be of survival value in sudden exposures to low salinity. It has, therefore, seemed of interest to compare the overall tendency to lose salts in selected species of brackish-water polychaetes, in order to prepare for more detailed studies aimed at evaluating the separate roles of body wall, nephridia, and gut in this process. The present studies have been directed to the working out of methods for comparing salt-loss rates, and advantage has been taken of an opportunity to compare worms from certain widely-separated localities.

MATERIAL AND METHODS

Three species of brackish-water nereid polychaetes have been used in these comparisons:

1) *Nereis diversicolor* O. F. Müller, an extremely widespread species whose range includes brackish and estuarine waters in northwestern Europe, where it may penetrate into extremely low salinities, but does not live in fresh water except temporarily (Smith, 1955).

2) *Nereis limnicola* Johnson, a species found in estuaries on the west coast of North America, and in the fresh-water Lake Merced at San Francisco. It is closely related to *N. diversicolor* (Hartman, 1960; Smith, 1958), but is unique

in being viviparous. *Neanthes lighti* Hartman is a synonym. It should be noted that *N. diversicolor* and *N. limnicola* are closely related, even being considered conspecific by Hartman (1960), although other workers (Smith, 1958; Khlebovich, 1963) prefer to regard them as separate species. Both are referred to the genus *Neanthes* by Hartman, but this usage has not been uniformly accepted.

3) *Nereis* (*Neanthes*) *succinea* Frey and Leuckart, is widespread (Smith, 1963) both in Europe and America in brackish waters, but does not penetrate into such low salinities as does *N. diversicolor*. *N. succinea* is the same as the *Nereis limbata* Ehlers used in embryological studies at Woods Hole. It, too, is often referred to as *Neanthes*, especially in American usage. It should suffice for experimentalists that *N. succinea* stands somewhat apart from *N. diversicolor* and *N. limnicola*, and, unlike them, forms a reproductive swimming stage or "heteronereid." In the present study only its sexually immature "nereid" form has been used.

N. diversicolor has been studied at Kiel (Germany) near the mouth of the Baltic Sea, where the salinity approximates 50% sea water and at Turku (Finland), near the limits of its ecological range in the inner Baltic. *N. limnicola* has been collected from the relict fresh-water Lake Merced in San Francisco, and from the estuary of Walker Creek, emptying into Tomales Bay, California. *N. succinea* has been studied briefly at Kiel, and in more detail at Berkeley, using specimens taken in south San Francisco Bay, where salinities vary from 50% to 85% of sea water.

In order to have a standard basis for comparison of different nereid species in respect to their tendency to lose salts, it is necessary to set the experimental conditions rigidly, and the following procedures have been adhered to as closely as possible:

1. In order to assure the same osmotic or concentration gradient, worms are adapted before testing in sea water diluted to a chlorosity of 10 g./L., which is approximately equivalent to 55% of sea water and has a conductivity roughly that of 0.3 molar NaCl. At such a concentration, these worms are above the regulatory range and essentially conform to the chlorosity and osmotic concentration of the medium.

2. The test of salt loss rate is made immersing the worms (after a brief rinse) in a measured volume of glass-distilled or de-ionized water for one hour, at the end of which the conductivity of that water is measured and the salts lost calculated as the equivalent of NaCl or KCl. The volume of distilled water used was 1 ml. per 2.5 mg. wet weight of worm, and the concentration of salts lost was so low that recovery by active uptake could be disregarded. (Note: Metal-distilled water is toxic, resulting in permanent injury, and must be avoided.) The one-hour exposure in these experiments caused great swelling in *N. succinea*, but recovery appeared to be complete after return to the adaptational medium.

3. Since salt loss is undoubtedly taking place through external body surfaces as well as via nephridia and possibly gut, it is to be expected that it would be related both to body weight and to surface. The data obtained under the specified conditions have been plotted as straight lines on a double log plot, according to the common expression: Loss = a (Weight)b where "Loss" is the millimoles of salt lost per worm per hour; "Weight" is the wet weight in milligrams taken before

exposure to distilled water; "b" represents the slope of the line in the double log plot, and "a" the intercept at the y-axis when x = unity. Expression of results in this fashion permits comparison between species and populations differing in mean size and size range, as is essential in working with such animals, since it is generally impossible to collect worms of uniform or specified size in sufficient number, or to find populations of the same mean size in different localities. Considerable scatter has been found in these studies, which may in part be caused by variation in the amount of water held in the gut, although all worms were kept about a day in clean water to allow the emptying of sand and fecal material. Attempts to use dry weight did not, however, produce detectably less scatter.

4. Salt loss in distilled water should be carried out at a uniform temperature; this has unfortunately varied somewhat in different laboratories: the room temperature was 18–21° C. at Kiel; controlled baths at 18° C. in Turku, and at 15° C. in Berkeley, were used. Standard conductivity bridges (Siemens, Philips, and Industrial Instruments, Inc.) have been used. A blood-pipette conductivity cell was used at Kiel, a Philips dip cell at Turku and Berkeley. Conductivities have been expressed as equivalent millimoles of monovalent salt (NaCl or KCl) read from calibration curves constructed for the temperature and the cell used in each laboratory, and the salts lost by each worm in the known volume of water calculated.

It is clear that this type of comparison is based upon a highly unnatural type of exposure of the worm to minimum salinity. The tests are, in fact, a standardized osmotic emergency. The plunging of a worm into salt-free water not only results in salt loss but also water entry, and initiates responses of osmotic and volume regulation. The rate of salt loss declines with time; hence even an exposure as short as one hour gives too low a value for the rate existing at the start of the exposure; this fact is of lesser importance when comparing *N. diversicolor* and *N. limnicola,* since the drop in salt loss rate during one hour is not very great (*i.e.,* the loss curve does not markedly decrease in slope), but it is undoubtedly important in the case of *N. succinea,* in which the osmotic gradient is greatly lowered by swelling during one hour, so that the loss rate as calculated over one hour must be lower than the initial rate. This limitation has been accepted for this preliminary survey, since it represents an error on the conservative side.

In addition to experiments involving the standard test procedure indicated above, some experiments have been performed on worms adapted to sea water dilutions of different chlorosity (1, 5, 15 and 18.6 g./L.), and in some instances isotonic solutions of non-electrolytes have been used instead of glass-distilled water in an effort to clarify certain problems. These will be mentioned below, in context.

Experimental Results

a) Inter-specific comparisons of salt loss rates

In Figure 1 are curves for the salt-loss rates of the three species, each represented by two populations. Inspection indicates that *N. succinea* loses more salt under the conditions of this experiment than do the other two species, which, as
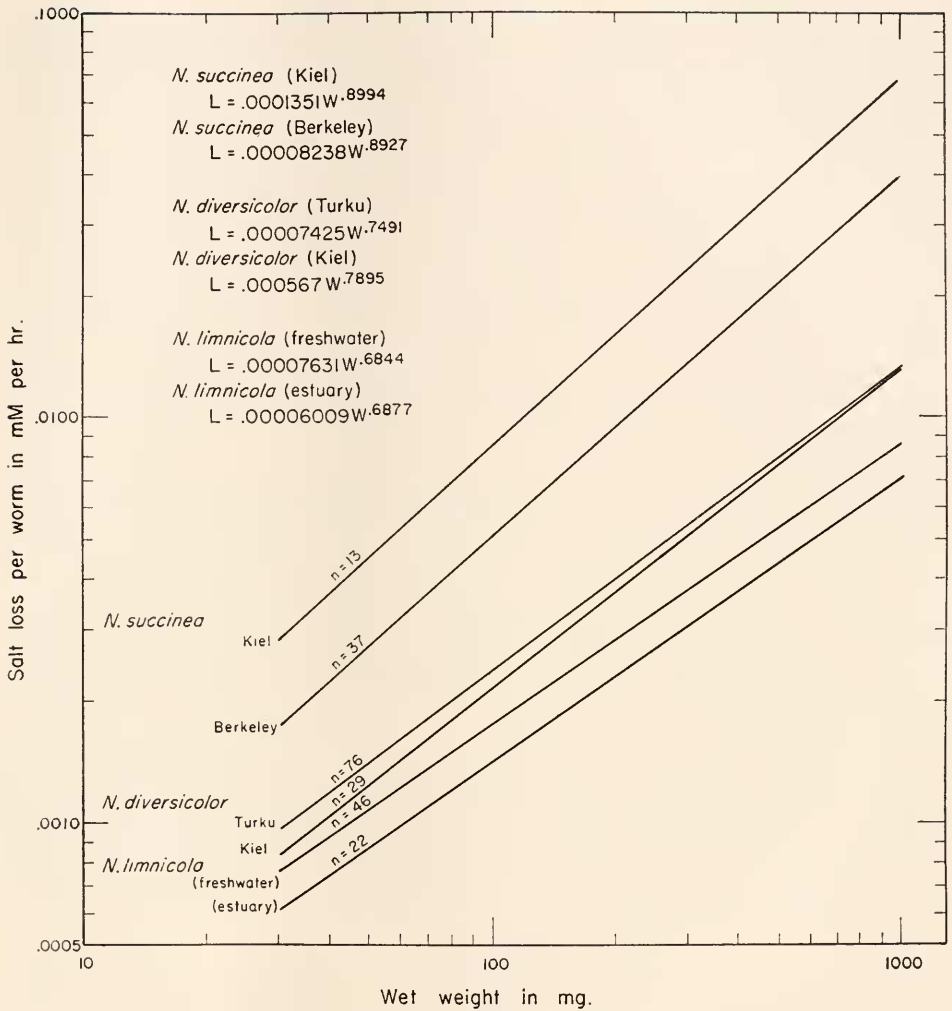
FIGURE 1. Salt loss per worm as a function of body weight under the standard conditions described in text.

expected, are more similar to each other. The salt losses per worm weighing one gram, as read from the curves, are:

N. succinea (Kiel)	0.068	mM/hr.
N. succinea (Berkeley)	0.039	mM/hr.
N. diversicolor (Kiel)	0.0129	mM/hr.
N. diversicolor (Turku)	0.0131	mM/hr.
N. limnicola (Lake Merced)	0.0086	mM/hr.
N. limnicola (Walker Creek)	0.0070	mM/hr.

The values for *N. succinea* show it losing 3 to 5.2 times as much salt per gram as *N. diversicolor*, which in its turn loses 1.5 to 1.9 times as much as *N. limnicola*.

For reasons mentioned above, the tendency of *N. succinea* to lose salts is actually greater than these curves indicate. The general height of these curves indicates that lessened salinity of the habitat is associated with a lesser rate of salt loss when the species are compared.

Not only do the salt loss curves differ in height between *N. succinea* and the other two species, but the slopes (b) are markedly greater in the case of *N. succinea* (0.899 and 0.893) than in the case of *N. diversicolor* (0.749 and 0.790) and *N. limnicola* (0.684 and 0.688). Values as low as 0.68 suggest that *N. limnicola*'s salt loss rate approximates the "surface rule." This may mean that this species does not change shape as it grows (a view that inspection verifies in a subjective way), and hence suffers the expected decrease in surface/volume ratio with increase in size. On the other hand, *N. succinea* seems to depart markedly from the "surface rule," and, although it does not attain a "b" value of 1.0, which would mean that it succeeded in keeping its surface/volume ratio constant during growth, its "b" value or slope of 0.89–0.90 suggests that it does change shape in such a way as to lessen the decrease in its surface/volume ratio during growth, a view subjectively confirmed by inspection of the conspicuously elongated posterior dorsal parapodial lobes developed by older and larger worms. Until a quantitative measure of surface area is available, this is the simplest explanation of the steeper slope of the salt loss curves of *N. succinea*. Since the curves for *N. succinea* would not cross those of the other two species unless extrapolated to unreasonably small weights, it is felt that the observed difference in height of the curves within the observed range of weights (roughly 30 to 1000 mg.) is the result of actual differences in outward permeability to salts rather than solely due to a greater surface/volume ratio in *N. succinea*.

Measurement of salt loss at short intervals shows that salt loss in these worms follows an expected course, dropping with time, as would be expected for two reasons, (1) that the gradient decreases because of salts passing out, and (2) that the gradient is lowered by dilution of the internal salts by water entering osmotically. The average percentages of swelling observed in the three species during the one-hour exposure to distilled water are:

N. succinea (Kiel)	50–60%
N. succinea (Berkeley)	57%
N. diversicolor (Kiel)	24%
N. diversicolor (Turku)	25%
N. limnicola (San Francisco)	10%

In the case of *N. succinea*, a net gain of over 50% the initial weight caused by water entry must have diluted the coelomic fluid enough to lower the salt gradient by at least half. At the same time, it may have had the effect of increasing the urinary output. Since the effect would have been simultaneously to reduce salt loss *via* the body wall and to increase it *via* the urine, these data do not permit one to state whether the recorded salt loss has been increased or decreased by the fact of osmotic water entry. An absolute value for urinary water output and salt content would be needed to answer this question, as well as information on the space occupied by the incoming water in the hour of exposure. Similarly, the lesser salt losses of *N. diversicolor* and *N. limnicola* cannot be attributed to their lesser degree of osmotic swelling, and we cannot say whether the smaller swelling

represents a smaller amount of water entry, or a more effective disposal of the water as urine. The latter two species, as compared to *N. succinea:* (a) are clearly more effective volume-regulators, remaining active and normal in appearance during the exposure, whereas *N. succinea* becomes swollen and motionless (although capable of quick recovery); (b) are more conservative of salts in the sudden exposure to fresh water, although it is not apparent whether low outward salt permeability or effective salt retention in nephridia is involved; and (c) show a decrease in salt loss rate with increase in body size, seemingly having the advantage of the "surface rule" as a consequence of constant body shape during growth.

The curves of salt loss rate in Figure 1 are in the order of decreasing salt loss with decreasing salinity of habitat of the three species involved. *N. diversicolor* at Kiel (habitat chlorosity about 10 g. Cl/L.) and at Turku (habitat chlorosity about 3 g. Cl/L.) behave almost identically. *N. succinea,* although overlapping the salinity range of *N. diversicolor,* is characteristically in a more saline habitat and does not tolerate such low salinities. At present no explanation of the higher salt loss rate observed at Kiel can be offered. The number of worms used at Kiel was small, and the possibility of experimental error in making up the adaptational media is not excluded. The curves for the fresh-water and the estuarine populations of *N. limnicola* seem paradoxical in that the salt loss is greater in the fresh-water group. There are probably two reasons for this: (1) Studies of the level of chloride regulation in *N. limnicola* (Smith, 1959) have shown that the Lake Merced population regulates its coelomic chloride above the level in the Walker Creek estuarine population, and even holds it slightly above the medium at a chlorosity of 10 g. Cl/L.; thus the salt gradient for the fresh-water population may have been slightly higher. (2) Studies on volume control (Smith, 1959) likewise showed that the fresh-water population of *N. limnicola* consistently exhibited a lesser swelling and a faster return to normal volume after transfer from sea water of chlorosity 10 g. Cl/L. to fresh water. This difference in response was not eliminated by over 60 days of adaptation of fresh-water and estuarine worms to identical salinities in the laboratory. The fresh-water population of *N. limnicola* is able to eliminate water faster than its estuarine relatives; presumably the extra urine discharged, at least in the early stages of adjustment in distilled water, eliminates salts and contributes to the greater salt loss rate of the fresh-water population. Thus the greater salt loss rate is seen as an advantage in rapid adjustment to the osmotic emergency.

b) Salt loss in N. diversicolor *as a function of the gradient*

In the inter-specific comparison just made, *N. succinea* showed a higher "b" value, which is thought to be a consequence of an increase in relative body surface during growth, causing a departure from the "surface rule." However, it might be asked if this high value of "b" is not in some way related to the very great swelling endured by *N. succinea* during the hour in distilled water. Although this view seems unlikely because the surface/volume ratio would be *decreased* by swelling rather than increased (note that the surface of a swelling nereid can unfold like an accordion, rather than stretching like a balloon), it may be tested experimentally by comparing the "b" values of the salt loss curves obtained under conditions in which the amount of swelling is varied. This experiment was done

using *N. diversicolor* at Turku. Groups of worms were adapted to dilutions of sea water of chlorosities 1, 5, 10, 15 and 18.2 g. Cl/L. prior to the standard one-hour exposure in distilled water. In Figure 2 are plotted the percentages of swelling as a function of the adaptational chlorosities. As expected, worms with the higher salt gradient swelled more. Note that worms adapted to 1 and 5 g. Cl/L. behaved in this respect rather similarly; these chlorosities lie in the "regulatory range," so that the internal concentrations are much the same. The worms adapted at 1 g. Cl/L. even showed a decrease in volume when placed in distilled water. This effect has often been noted, and probably reflects an increase in urinary output caused by increased activity and muscular tension, as postulated by
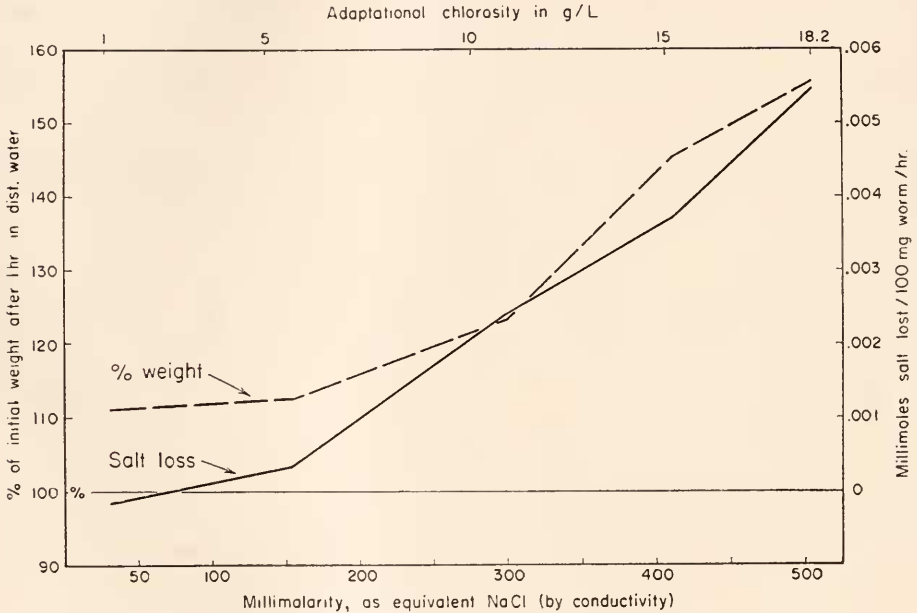

FIGURE 2. Relationship between the percentage of swelling (left-hand ordinate), the amount of salt loss per 100 mg., from curves in Figure 3 (right-hand ordinate), and the chlorosity and approximate millimolarity (equivalent NaCl) of the media to which *N. diversicolor* were adapted prior to one-hour exposure to de-ionized water.

Beadle (1937) in relation to volume control. Likewise, in Figures 2 and 3 it may be noted that salt loss in the worms adapted to 1 and 5 g. Cl/L. is almost identical, suggesting that the amount of salt lost in these experiments is a function of the gradient existing between interior and the medium. However, the "b" values of the set of curves in Figure 3 lend no support to the idea that the high "b" values for *N. succinea* could have been a result of excessive swelling. Rather, they suggest that volume increase merely depresses the surface/volume ratio. The low "b" values of 0.44 and 0.53 seen in the worms from chlorosities of 18.2 and 15 g. Cl/L. lend credibility to the supposition that the surface of a swelling nereid does not increase in area but merely unfolds as the worm takes in water. On this view, the extensive surface developed by a large *N. succinea,* even in the "nereid" form,
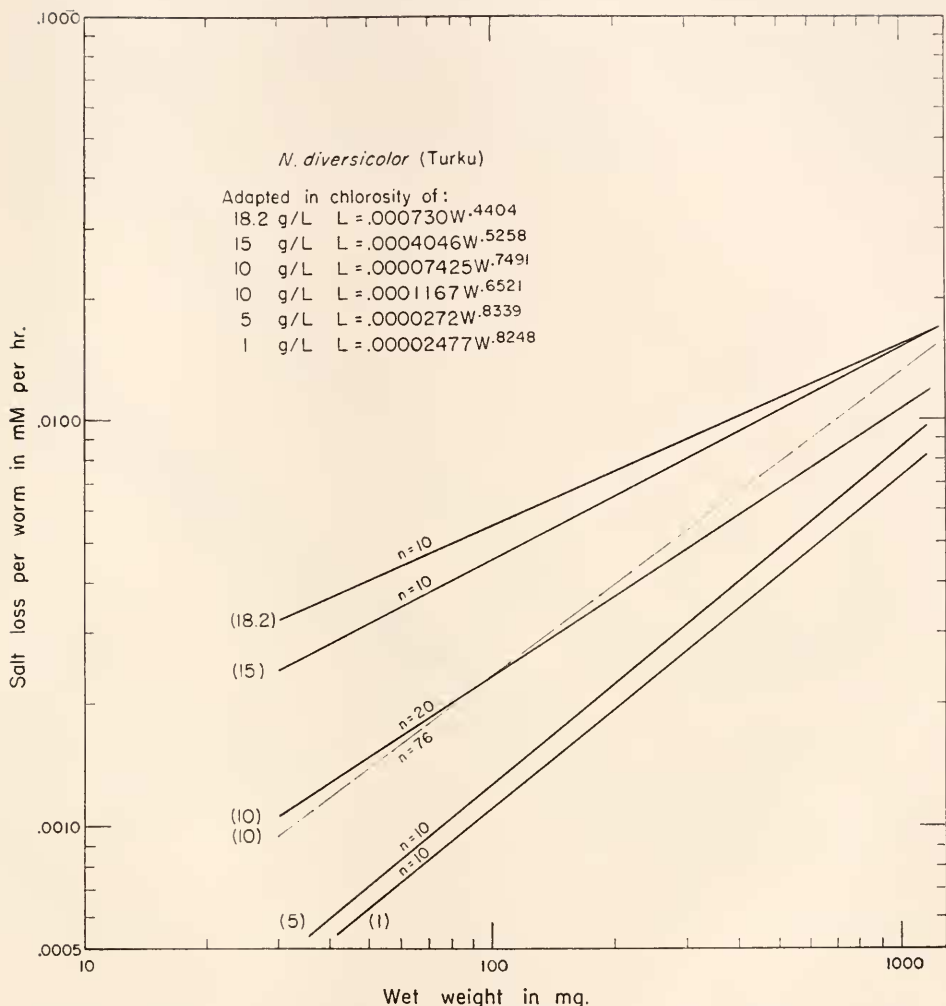
FIGURE 3. Salt loss per worm as a function of body size following adaptation to different chlorosities of diluted sea water. Note the differences in slope associated with the osmotic gradient and degree of swelling in the standard exposure to distilled water (see also Figure 2).

becomes a factor of importance in determining its lesser ability to retain salts and resist swelling when exposed to low salinities.

c) The effect of preventing osmotic water-entry upon salt-loss rates

The results of adapting *N. diversicolor* to various concentrations of sea water before the acute test of salt loss have indicated that the rate of salt loss to distilled water is a function of the salt gradient. However, since this gradient must be lowered by water entry during swelling, it was thought that if swelling could be prevented by the use of isotonic salt-free solutions rather than distilled water in
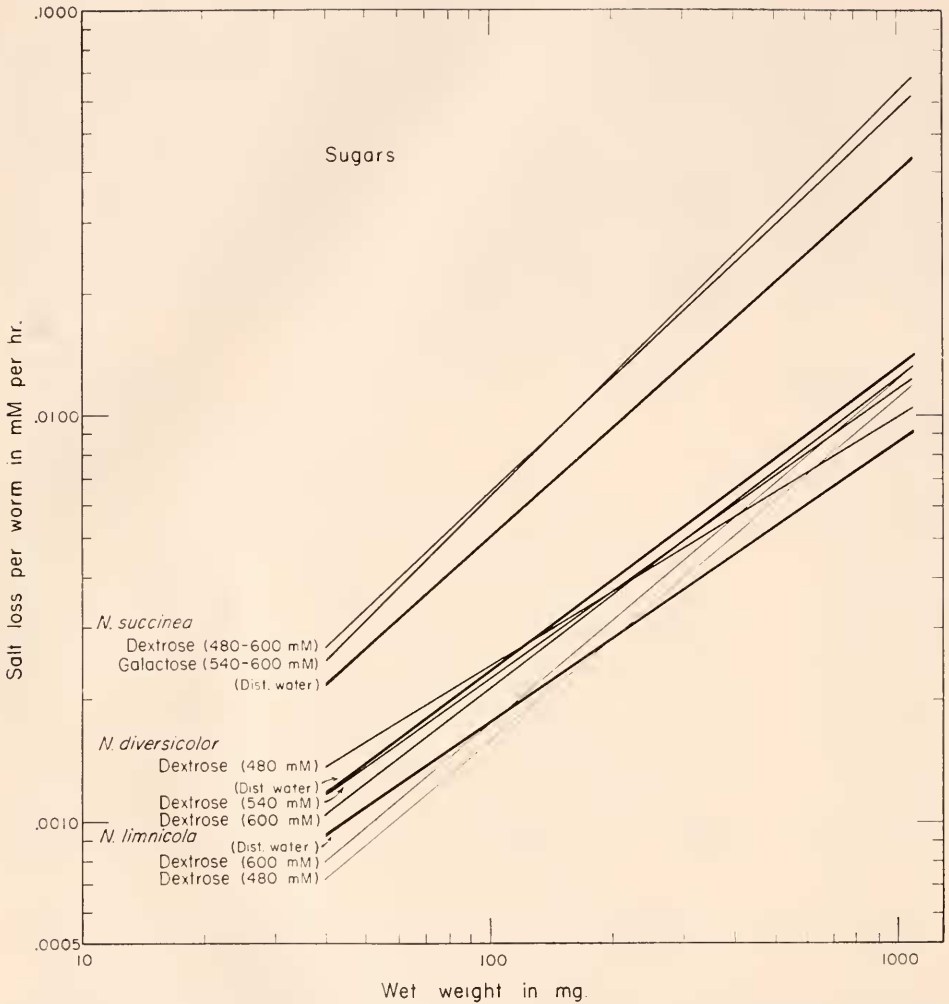
FIGURE 4. Typical examples of salt loss into isotonic (600 mM) or moderately hypotonic sugar solutions after adaptation to sea water of chlorosity 10 g./L. Least change of volume usually in approximately 90% of isotonic value. Control curves (distilled water) are from Figure 1. Loss is slightly increased from *N. succinea,* but no effect is demonstrable in *N. diversicolor* or *N. limnicola.* All curves from conductivity measurements.

the acute salt-loss tests, the salt loss rate might thereby be increased. Accordingly, a number of experiments have been made in which isotonic solutions of dextrose, sucrose, or galactose have been substitute for distilled water in standard salt-loss determinations. The results (Fig. 4) reveal no increase in salt loss rate in *N. diversicolor* or *N. limnicola,* species in which the extent of osmotic swelling in distilled water is slight. It has been consistently noted that a sugar solution isotonic to the adaptational sea water causes some loss of volume during the exposure; this volume loss is attributed to output of urine while osmotic water

inflow is stopped. Commonly, such worms swell when returned to the original media, suggesting that a concentration of the body fluids has occurred; this hypothesis would seem to require that the urine be hypotonic to the body fluid. Sugar solutions of 85–90% the osmotic pressure of the adaptational sea water caused very little volume change, suggesting that they allowed a net osmotic inflow equal to the urinary output.
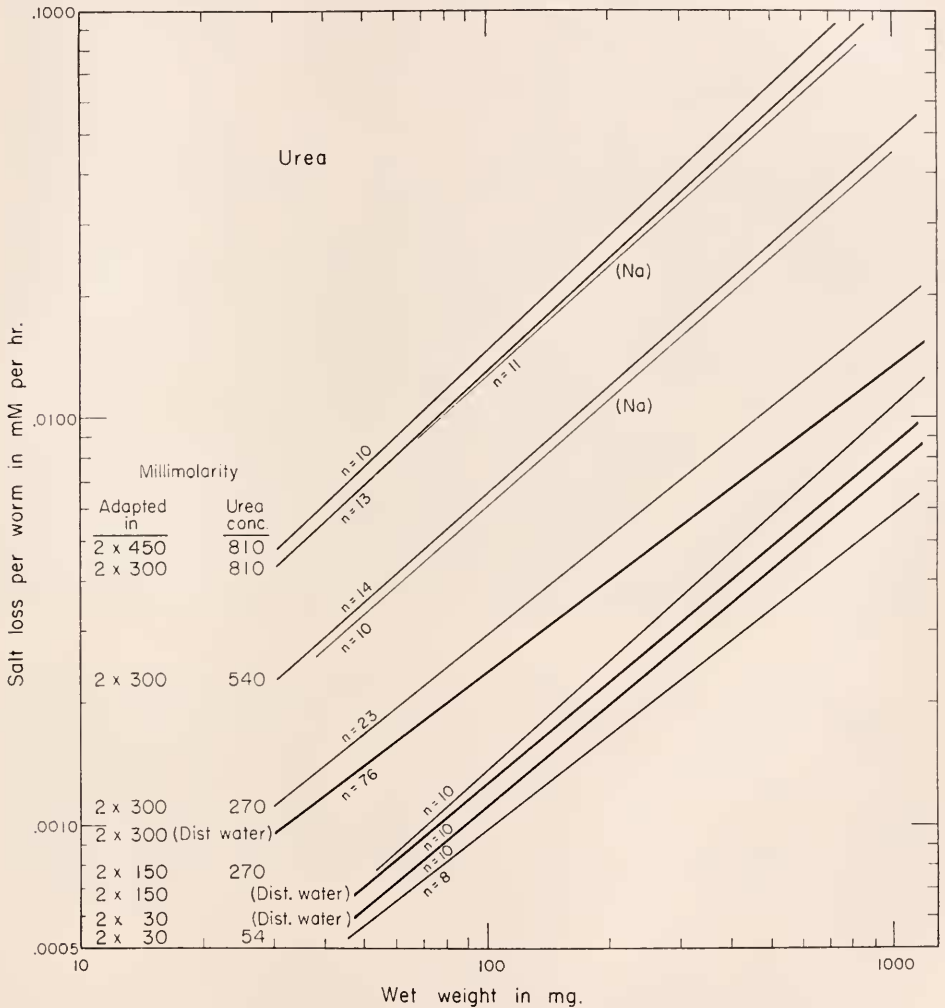

FIGURE 5. Salt loss into salt-free urea solutions, after adaptation to sea water diluted to 15, 10, 5 and 1 g. Cl/L., equivalent to NaCl concentrations of 450, 300, 150 and 30 mM, urea molarity set in relation to 2 × salt molarity. Note especially that salt loss level after adaptation to 10 g. Cl/L. (approximately milliosmolarity 2 × 300) increases with urea concentration, probably as an injury effect. Thin lines marked (Na) are calculated as 2 × the Na-millimolarity as determined by flame photometry; the rest by conductivity.

In the case of *N. succinea,* such sugar solutions seemed to cause a slight increase in salt loss rate, probably attributable to the maintenance of the salt gradient as a result of preventing dilution of the body fluids. The effect is not, however, very great. It seems to suggest that in *N. succinea* the maintenance of the salt gradient increases the outward passage of salts, and since the urine flow is probably not increased, this increased salt loss may be *via* the body wall. This latter point is, however, only inferred, not proved.

Surprisingly, even though salt-free sugar solutions prevented swelling and increased the salt loss only slightly (*N. succinea*) or not at all, the final condition of all three species after an hour in such solutions was clearly worse than in distilled water. They became motionless and often showed protruded pharynges; the anterior part of the body might be contracted and the head region flexed dorsally. Although recovery seemed complete, the impression was unavoidable that the sugar solutions were not physiologically neutral, and that the prevention of swelling was not free of side effects, the nature of which is not known. Possibly there is some penetration or active uptake of sugar into the body, although the fact that dextrose, galactose, and sucrose all had similar effects renders this explanation unlikely. Possibly the reduction of normal water fluxes leads to an ionic imbalance. Although the effects described merit further study, the use of isotonic solutions of non-electrolytes did not seem to contribute to clarification of the comparative performances of the species under study, and was discontinued.

Isotonic solutions of urea were also tested, upon *N. diversicolor* only. In all cases, urea solutions increased the salt loss rate, but here the rate of salt loss seems related to the concentration of urea applied, rather than to the salt concentration gradient (Fig. 5). The results are explicable on the basis of increased outward permeability to salts caused by the urea in proportion to its concentration. The worms recovered from short exposures, but exposures of an hour or longer were clearly deleterious. Since experiments with urea evidently involved damage to the body surface, they also were discontinued.

Summary

1. A standardized method is presented for measuring and comparing the total salt loss rates of different species of brackish-water polychaetes acutely exposed to an osmotic emergency situation.

2. *Nereis* (*Neanthes*) *succinea* shows a high salt loss rate in comparison to *Nereis diversicolor* and its close relative *N. limnicola.* These rates decrease in proportion to the degree of salinity lowering tolerated by these species in their natural habitats.

3. In *N. diversicolor,* the rate of salt loss is proportional (under the standard conditions) to the concentration gradient between body fluid and outside medium.

4. In *N. limnicola,* the tendency of the fresh-water population to have a higher salt loss rate than an estuarine population is seen as a result of a higher level of salt regulation and a more effective volume regulation by urinary output.

5. The slopes of the salt loss curves suggest that *N. succinea* changes shape during growth in such a way as to lessen the expected decrease in its surface/volume ratio, and thus departs from the "surface rule." *N. limnicola* (and, to a lesser extent, *N. diversicolor*) does not seem to change shape during growth and follows the "surface rule" in respect to rate of salt loss.

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